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ANTIOXIDANT EFFECT OF ETHANOLIC LEAF EXTRACT OF *PIPER GUINEENSE* IN ALUMINIUM CHLORIDE INDUCED REPRODUCTIVE TOXICITY AND OXIDATIVE STRESS IN MALE ALBINO RATS.

Arhoghro E. M.[1]* and Sule O. J.[1]

[1]Department of Biochemistry, Niger Delta University, Bayelsa State, Nigeria.

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***Corresponding Author**
Arhoghro E. M.
Department of Biochemistry,
Niger Delta University,
Bayelsa State, Nigeria.

ABSTRACT

Aluminium is one of the highly abundant element in the environment and the and the third most common metal in the earth crust. *Piper guinense* is herbal medicine used for the treatment of many diseases like infertility. This study shows the antioxidant effect of *Piper guineense* on infertility caused by aluminium chloride in male wistar rats. Male wistar albino rats weighing between 150-200g was randomly separated into five different groups. The first group served as negative control and received distilled water and standard feed. Group two served as positive control and received $AlCl_3$ (4.3mg/kg bw) and Group three received $AlCl_3$ (4.3mg/kg bw) and *Piper guineense* (100mg/kg bw) for 21 days. There was a significant ($P<0.05$) decrease in the activities of SOD, CAT, GPX, GST, GSH and there was a significant ($P<0.05$) increase in LPO from, in rats treated with $AlCl_3$ (4.3mg/kg bw) compared to the negative control.Also there was a significant ($P<0.05$) increase in the activities of SOD , CAT, GPX, GST and GSH in rat testis treated with *Piper guineense* (100mg/kg bw) plus $AlCl_3$ (4.3mg/kg bw) compared to the positive control while there was a significant ($P<0.05$) decrease in LPO from$12.75 \pm 0.521$to 9.02 ± 0.439 in rat's testis treated with *Piper guineense* (100mg/kg bw) plus $AlCl_3$ (4.3mg/kg bw) compared to the positive control. There was a significant ($P<0.05$) increase in activities of ALT and AST, in rat serum treated with $AlCl_3$ (4.3mg/kg bw) compared to the negative control and there was a significant ($P <0.05$) decrease in ALT and AST, in rat serum treated with *Piper guineense* (100mg/kg bw) and $AlCl_3$ (4.3mg/kg bw) compared to the positive control. The results obtained indicate that *Piper guineense* have very high protective properties against reproductive toxicity, this high level of antioxidant activities present in the plant alters the

toxic effect of aluminium chloride which induces depletion of the defence system in the testes.

KEYWORDS: *Piper guineense* Aluminium chloride biochemical parameters, oxidative stress.

INTRODUCTION

Humans are inevitably exposed to metals due to their ubiquity in nature, contaminated air, water, soil and food, wide use in industry and long-term persistence in the environment. Metals may have serious effect on the male reproductive system directly, when they target specific reproductive organs, or indirectly, when they act on the neuroendocrine system. Metals have been shown to affect spermatogenesis in rodents and humans, which can lead to low sperm count, abnormal sperm morphology and poor semen quality,[1,2] among them Aluminium (Al) is the most widely distributed metal in the environment. Some of the factors responsible for this infertility are associated or traced to hormonal secretion, erectile impotency, disorders of ejaculations, toxic effects of substances on testes and accessory sex organ.[3] Over the years, plant extracts and plant-derived medicines have made immense contributions to the overall health and well being of human beings.[4] The use of plants as medicine by people dates as far back as the beginning of civilization. Plants are important sources of many biologically active compounds. Plants used in traditional medicine provide an interesting and still largely unemployed source for the development of new drugs.[5] Globally about 85% of all medications for health care are derived from plants.[6] Medicinal plants have various effects on living systems.Some are sedatives, analgesics, antipyretics, cardioprotectives,antibacterials, antivirals and antiprotozoals. However, this study focuses on remedies for fertility.

Piper guineense is a West African species of pepper; the spice derived from its dried fruit is known as West African pepper, Ashanti pepper, Benin pepper, False cubeb, Guinea cubeb, Uziza pepper, or guinea pepper and called locally Kale, Kukauabe, Masoro, Sasema, and Sorowisa.[7]

Piper guineense is a perennial woody climber that grows up to 10 m or more in height. Its leaves are alternate and simple with a petiole 2–5 cm long. It is a plant of the wet tropics that requires heavy and well distributed rainfall and temperature. The ideal soil for production is a well-drained alluvium with high humus content. It can also be grown in red laterite soil. The

leaves of *Piper guineense* are used as a leafy vegetable pepper in most of the African soups. The leaves and fruits are also used as flavor in most dishes. Traditionally, people believe that they have medicinal properties. *Piper guineense* is added to food meant for pregnant and nursing mothers as a medicinal spice and among the postpartum women. It is claimed that it assists in the contraction of the uterus.[8]

The seeds, leaves, and sometimes the stems are used in preparing soup. It imparts "heat" and a spicy pungent aroma to food. The medicinal properties of *Piper guineense* exert bacteriostatic and bactericidal effects on some bacteria. The leaves are considered aperient, carminative, and eupeptic. They are also used for the treatment of cough, bronchitis, intestinal disease, and rheumatism. The leaves are also used for management of female infertility while the fruits are used as an aphrodisiac.[7]

The present study was done to delineate the influence of aluminium chloride on enzymatic antioxidants and ethanolic leaf extract of *Piper guineense* on the testes of rats.

MATERIALS AND METHODS

1 Animals

Adult healthy 15 male wistar albino rats weighing between 150g to 200g were obtained from the animal house of Niger Delta University Bayelsa State and were acclimatized for two weeks during which they were feed with standard feed (pellet) and distilled water. All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the direction of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA).

2 Chemicals

Ethanol (EmerekDarstaatm.w 46.07), Aluminum chloride (BDH chemical Ltd. England).Kits from Randox Laboratories Ltd, Co. Antrim, United Kingdom.Sigma-Aldrich Ltd., U.S.A. PerkinElmer, USA were used. All other reagents/chemicals obtained from standard suppliers were of analytical grade.

3 Preparation of extracts

Piper guineense leaves were gotten in Amassoma market, Bayelsa state and washed with clean water air dried at room temperature.The plant was botanically identified and deposited at the Herbarium of department of biological science, in Niger Delta University (N.D.U),

Amassoma, Wilberforce Island, Bayelsa State, Nigeria.. The dried leaves were ground into powder with the aid of a grinding mill machine and stored in an air tight container to prevent microbial growth.

The *Piper guineense* powder was mixed with ethanol in ratio 1:3 using a wooden spoon to continuously stir at interval for two days (48 hours). Then the mixture was filtered using a filter paper and the filtrate was evaporated to dryness in a water bath for two days at 60^{o}C to obtain a paste. Appropriate weights of the residue were prepared in distilled water to obtain various concentrations that were administered orally to each of the rats.

4 Experimental design and procedures

4.1 Experimental design

A total of 15 adult albino rat strains grouped into three each having five rats.

Group 1Group 1: Negative Control (2ml/kg body weight (bwt) distilled water and pellet feed for 21 days.

Group 2 (positive control): Received Aluminum chloride [4.3mg/kg bwtintraperitonealy (i.p)] and feed with pellet feed and distilled water for 21 days.

Group 3 (experimental group 3): Received 100mg/kg (bwt) of *Piper guineense*extract orally, aluminum chloride [4.3mg/kg bwtintraperitonealy (i.p)] and fed with pellet feed and distilled water for 21 days. Aluminum chloride was administered 45 minutes after oral administration of *Piper guineense*.

4.2 Sample Collection and Biochemical Analysis

After the experimental period, animals in all groups were sacrificed. By the end of each experimental period, the rats were reweighed, starved for 24 hours and sacrificed under chloroform anesthesia. 5ml of blood was collected from each animal by cardiac puncture using sterile needle and syringe. Part of the blood sample was put into test tubes and allowed to clot for 30 minutes before centrifuging at 800g for 5 minutes. The supernatant was used for the biochemical analysis. The testes and epididymis were excised using a midline abdominal incision. The testes were immediately weighed and the epididymis transferred into sterile bottles containing 10ml of normal saline for semen analysis. The testes were also transferred into 10% neutral buffered formalin for histopathological examination. Testes were excised

and washed in cold saline, Ten percent tissue homogenates were prepared in 0.1M Tris –HCL buffer (pH 7.4).

4.3 Biochemical parameters.

a) **Enzyme analysis:** The following liver function test were conducted to investigate derangement in the liver of the animals used for the study, aspartate aminotransferase (AST), alanine aminotransferase (ALT) were determined by the colorimetric method of[9] using a commercial assay kit from Randox Laboratories Ltd, Co. Antrim, United Kingdom.

b) Markers of oxidative stress/ disturbances

Catalase activity was determined by the method of Cohen *et al*[10] Super oxide dismutase (SOD) activitywas measured by the method of Misra and Fridovich[11] The Gluthathione peroxidase (Gpx) activity was measured by the method of Chance and Maehly[12] as provided by Sigma-Aldrich Ltd., U.S.A. Glutathione-S-transferase activity was determined according to Habig*et al*.,[13] The assay method of Hunter *et al*.[14]as modified by Gutteridge and Wilkins[15] was adopted for the assay of Malondialdehyde (MDA) concentration

4.4 Histopathological study: Small pieces of testes tissues were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6µm in thickness were cut and stained with hematoxylin and eosin.[16]

5 STATISTICAL ANALYSIS

Data was expressed as mean ± SD of five estimations. The statistical significance was evaluated by one way ANOVA using SPSS (Statistical Package for Social Sciences) version 16.0 and the individual mean compared by post Hoc LSD and Tukey method. Values were considered statistically significant when $p<0.05$.

RESULTS

Table 1 Effect of aluminum chloride ($AlCl_3$) and ethanol extract of *Piper guineense* on antioxidant enzymes and lipid peroxidationin the testes of albino rats.

Enzyme	SOD (unit/mg protein)	CAT (unit/mg protein)	GST (unit/mg protein)	GPx (unit/mg protein)	LPO (unit/mg protein)
Negative control Distilled water	4.21 ± 0.18^a	5.19 ± 0.30^a	4.48 ± 0.27^a	6.51 ± 0.42^a	8.65 ± 0.47^a
Positive control ($AlCl_3$) 4.3mg/kg	3.35 ± 0.15^b	4.17 ± 0.25^b	3.15 ± 0.28^b	5.05 ± 0.36^b	12.75 ± 0.52^b

$AlCl_3$+*Piper guineense* 4.3mg/kg/bw + 100mg/kg	4.17 ± 0.18^c	5.07 ± 0.30^c	4.31 ± 0.26^c	6.40 ±0.42^c	9.02 ±0.44^c

Data are Mean ± SD (n= 5). Mean in the same column with different superscript letter(s) are significantly different, (P<0.05) one way ANOVA.

There was significant (P<0.05) decrease in SOD, CAT, GPx and GST, respectively and significant (P<0.05) increase in LPO in male albino rat testes treated with $AlCl_3$ compared to the negative control. On the other hand there was significant (P<0.05) increase in SOD, CAT, GPx and GST, respectively and significant (P<0.05) decrease in LPO in male albino rat testes treated with $AlCl_3$ and *Piper guineense*(100mg/kg bw) compared to the positive control respectively (Table 1).

Table2: Effect of administration of aluminum chloride ($AlCl_3$) and ethanolic extract of *Piper guineense* on serum AST and ALT of albino male rats.

Treatment	AST (U/I)	ALT (U/I)
Negative control (distilled water)	10.05 ± 1.00^a	12.50 ± 0.10^a
Positive control ($AlCl_3$) 4.3mg/kg	24.09 ± 3.40^b	18.30 ± 3.00^b
***Piper guineense* + $AlCl_3$ 4.3mg/kg +100mg/kg**	13.05 ± 1.30^c	13.20 ± 0.40^c

Data are Mean ± SD (n= 5). Mean in the same colum with different superscript letter(s) are significantly different, (P<0.05) one way ANOVA.

The result of AST and ALT activity in the serum showed significant (p<0.05) increase in rats treated with Aluminium chloride compared to the negative control, while there was a significant (p<0.05) decrease in AST and ALT activity in the serum in rats treated with *Piper guineense* and Aluminium chloride compared to the positive control (Table 2).

Histopathological findings. Testes from group 1 showed a normal feature of seminiferous epithelium and Interstitial tissue with active spermatogenesis (figure 1). Testes from those treated with aluminium chloride revealed a markedly shrunken seminiferous tubules with severe sperm cell aplasia and basement membrane thickening as well as rupture, vacuolization and fibrosis in interstitial and peritubular tissue (figure 2). Administration of ethanolic root extracts of *Piper guineense* along with aluminium chloride restored these changes towards normalcy (Fig. 3).

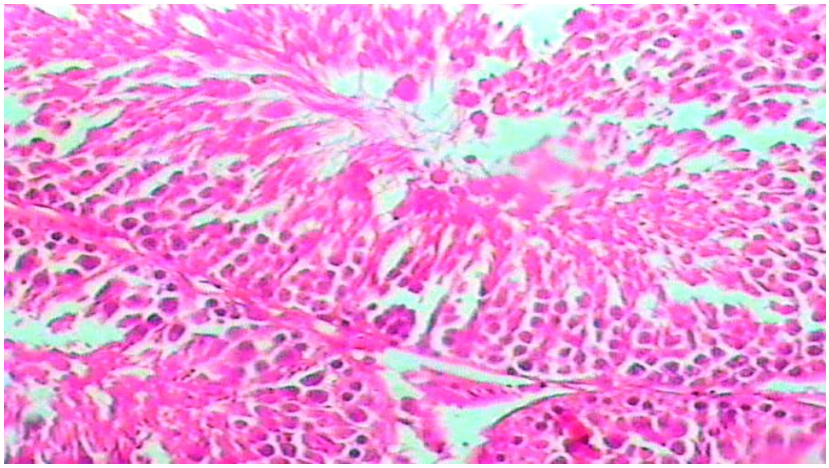

Fig. 1: Photomicrographs of testicular sections of control rats. Testes exhibiting a normal feature of seminiferous epithelium and interstitial tissue with active spermatogenesis

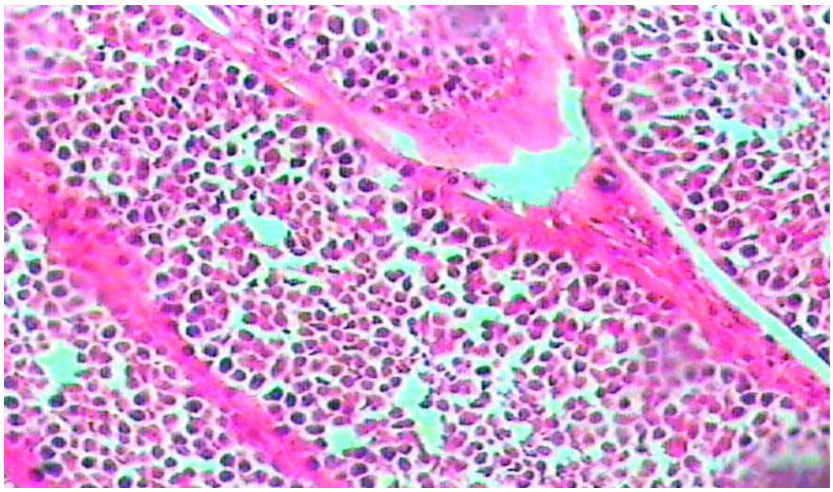

Fig. 2: Photomicrograph of testicular section of Aluminium chloride treated rats reveals markedly shrunken semeniferous tubules with severe germ cell aplasia and basement membrane thickening

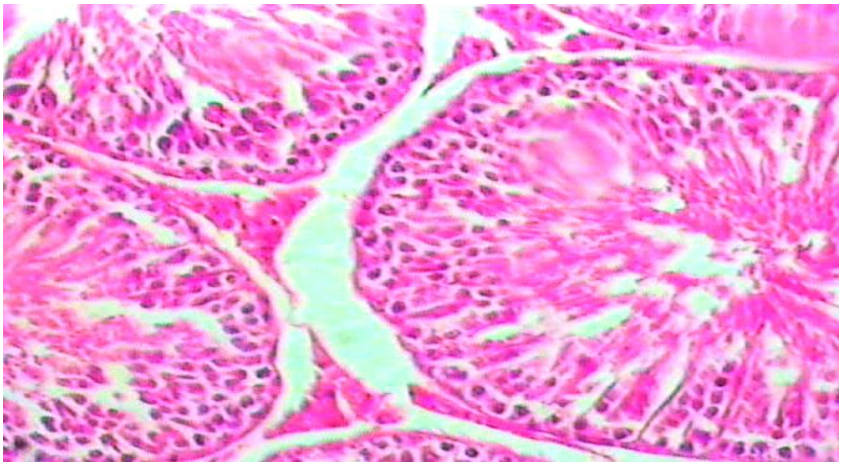

Fig. 3: Photomicrograph of testicular sectionof rats given ethanolic leaf extracts of *Piper guineense* along with Aluminium chloride atrophed seminiferous tubules with widen interstitial space, spermatogonia spermatids and spermatocytes.

DISCUSSION

Male fertility disorder is attributed to environmental factors such as exposure to certain chemicals, heavy metals, pesticides and heat or electromagnetic radiation.[17]

The serious social implications of infertility have made its prevalence a reason for public health interest in most developing nations.[18] Social, economic and personal effects which go beyond childlessness can be caused by fertility problems.[19] This is a major reason for marital problems in some locality.[20] Studies conducted on diets of natural origin like plantain has revealed that consumption could improve reproductive functions and also ameliorate certain reproductive dysfunctions.[21, 22] Arhoghro and Sule[23] also reported the ameliorative effect of *Rauwolfia vomitoria* in reproductive toxicity and oxidative stress induced by aluminium chloride in rats.

Cells are equipped with antioxidant defense system to counter the effect of relative oxidative stress (ROS). Environmental contaminants are known to induce reproductive toxicity by perturbing the pro-oxidant and antioxidant balance leading to oxidative stress.[24] Testicular oxidative stress appear to be a common feature in infertility which suggest that there may be benefits to develop better antioxidant therapies for relevant case of hypo spermatogenesis.[25,26]

In the present study which was carried out to elucidate the antioxidant effect of *Piper guineense* extract on aluminum chloride induced reproductive toxicity. Aluminum chloride treatment decreased the activities of the antioxidant enzymes SOD from 4.21 ± 0.18 to 3.35 ± 0.15, CAT, GP_X, GST and increases the activity of LPO from 8.65 ± 0.47 to 12.75 ± 0.52 in the testes compared to the negative control. While treatment of the plant *Piper guineense* and aluminium chloride increased the activity of the antioxidant enzymes SOD from 3.35 ± 0.15 to 4.17 ± 0.18, CAT, GST, GP_X and decreased the activity of LPO from 12.75 ± 0.52 to 9.02 ± 0.44 in the testes compared to the positive control (Table 1). Also aluminum chloride treatment increased the activity of AST from 10.50 ± 1.4 to 24.09 ± 3.40 and ALT in the serum of the rat compared to the negative control. While treatment of the plant *Piper guineense* and aluminium chloride decreased the activity of AST from 24.09 ± 3.40 to 13.05 ± 1.30 and ALT in the serum of the animals compared to the positive control (Table 2). The biochemical changes in the testes of rats administered aluminum chloride are similar with Sohier and Haya[27] who studied the effect of Balanitesa egyptiaca sapogenic extract on anti-infertility induced by aluminum chloride in male rats. Arhoghro and Sule[23] also reported the

ameliorative effect of *Rauwolfia vomitoria* in reproductive toxicity and oxidative stress induced by aluminium chloride in rats.

The increase in the activities of AST and ALT in serum of rats treated with aluminum chloride may be due to the leakage of these enzymes from the liver cytosol into the blood stream and/or liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane takes place. Aluminum exposure can result in aluminum accumulation in the liver and this metal can be toxic to the hepatic tissue at high concentrations.

The level of lipid peroxidation increased by the aluminum chloride administration clearly indicates an imbalance between pro-oxidant and antioxidant system, which could induce oxidative stress. The increase in lipid peroxidation in the testis, as observed in the present study, could be due to the concomitant increase in the generation of free radicals like hydrogen peroxide and hydroxyl radicals in these organs of aluminum chloride treated rats. Other studies have reported that ROS induce lipid peroxidation and the toxicity of lipid peroxides play a key role in the inhibition of sperm functions and the pathophysiology of male infertility.[28] The reduction in the activities of antioxidant enzymes could reflect the adverse effect of aluminum chloride on antioxidant system in the testis and of rats. The reduction in the activity of SOD causes a rise in the level of superoxide anion, which inactivates CAT activity. SOD is considered as the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen.[29] The decreased activity of CAT in the testes of aluminum chloride treated animals observed in the present study may reflect the inability of these organs to eliminate hydrogen peroxide produced by the influence of aluminum chloride. The antioxidant enzymes CAT and GP_X protect SOD against inactivation by hydrogen peroxide. In turn, SOD protects the CAT and GP_X against inhibition by superoxide anion.[29] Decreased activity of CAT could be associated with the oxidative stress in testis. CAT is the main scavenger of hydrogen peroxide at high concentrations. Along with CAT, GPx is also involved in the scavenging of hydrogen peroxide.[30] It is evident that ROS induced the tissue damage by initiating the self-propagating lipid peroxidation reaction.[31] The reduction in the activities of antioxidant enzymes in the testis could reflect the adverse effects of aluminum chloride on the antioxidant system of spermatozoa as well. Metal toxicity is considered to be one of the pro-oxidants that induce oxidative stress. ROS are important mediators of normal

sperm function and are involved in the induction and development of sperm hyperactivation, capacitation, and acrosome reaction.[32] However, excessive production of ROS above normal levels results in lipid peroxidation and membrane damage leading to loss of motility, damage to the acrosomal membranes and DNA oxidation, which render the sperm cell unable to fertilize the oocyte.[33]

The protective effect of *Piper guineense* on the antioxidant enzymes and bio markers (ALT and AST) against aluminum chloride is similar with effect of Balanitesaegyptiaca sapogenin extract against aluminum chloride induced infertility and dysfunction in rat testes studied by Soheir and Haya.[27]The result of this study are also in agreement with work by Arhoghro and Sule[23] who reported that ethanolic root extracts of *Rauwolfia vomitoria* ameliorates reproductive toxicity and oxidative stress induced by aluminium chloride in rats.

Histopathological examination of rats group administered $AlCl_3$ showed a markedly shrunken seminiferous tubules with severe sperm cell aplasia and basement membrane thickening as well as rupture, vacuolization and fibrosis in interstitial and peritubular tissue. Meanwhile, Administration of ethanolic extracts of *Piper guineense* along with aluminium chloride restored these changes towards normalcy. The histological changes in testes of rats administered AlCl3 are in agreement with Khattab[34] who studied the effect of AlCl3 on the rat's testes. Also, Guo*et al*.[35] observed deleterious effects and histopathological changes in testicular tissues after 2 weeks of aluminium treatment, as well as noticeable spermatogenetic loss as necrosis in the spermatids and spermatozoa at the 5^{th} week of aluminium treatment. This damage effect may be explained by Yousef and Salama[36] who reported that oxidative stress results from the production of oxygen radicals in excess of the antioxidant capacity of the stressed tissue. Many conditions or events associated with male infertility are inducers of oxidative stress, which leads to an increase in germ cell apoptosis and subsequent hypospermatogenesis, such stress condition, can cause changes in the dynamics of testicular microvascular blood flow, endocrine signaling, and germ cell apoptosis. Moreover, reactive oxygen species and oxidative damage of bimolecular may contribute to male infertility by reducing sperm function.[37]

This study provides scientific evidence that *Piper guineense* is a useful remedy against oxidative stress. *Piper guineense* has very high protective properties against reproductive toxicity.This high level of antioxidant activities present in the plant significantly

decreases($P<0.05$) the toxic effect of Aluminium chloride which induces depletion of the defense system in the testes. Furthermore, since *Piper guineense*is a common food spice among some West African nation like Nigeria, there is a strong need to carry out further studies on the effect of its extract on fertility of consumers of this plant.

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